Figure S1

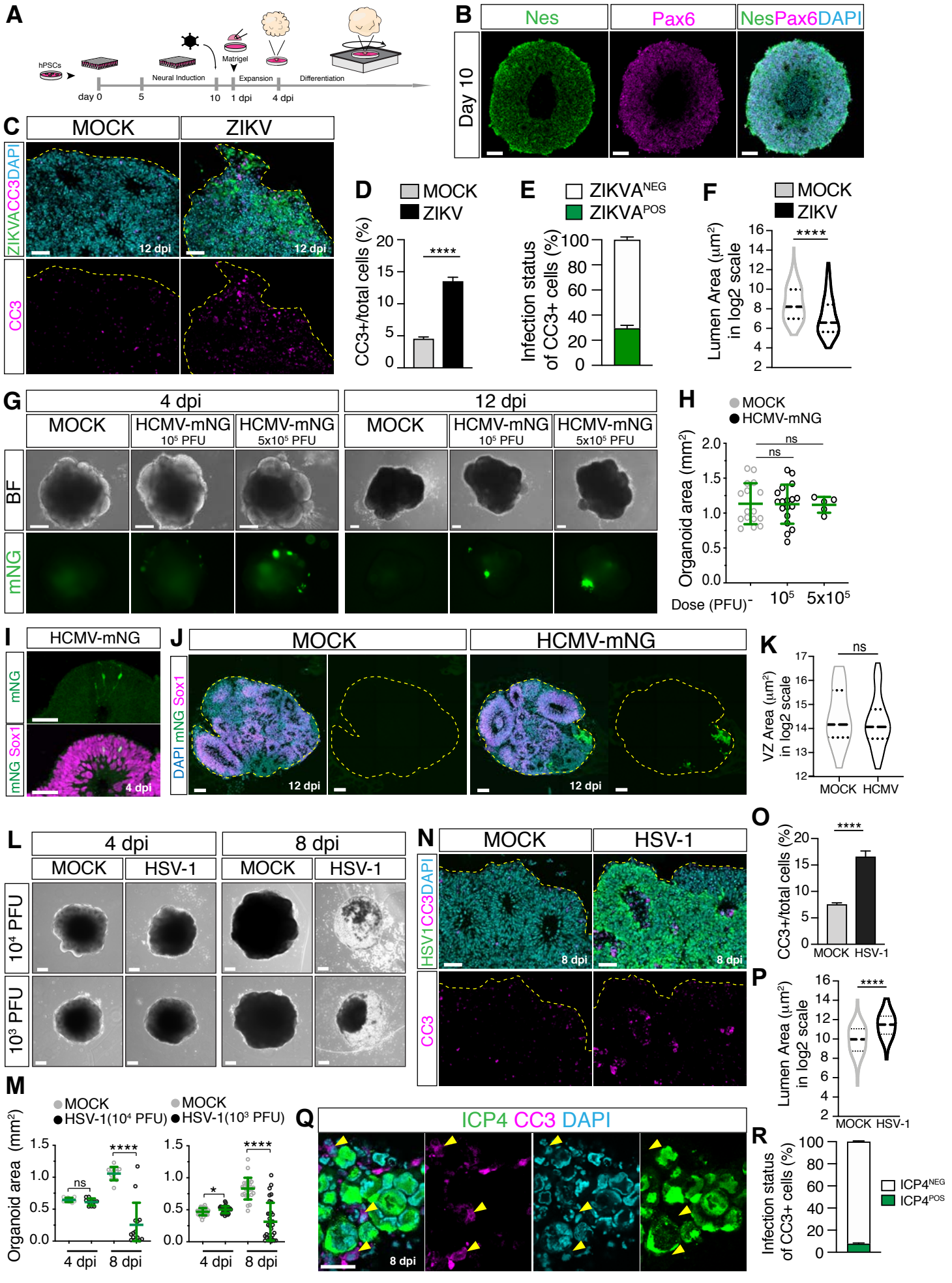

A
hPSCs
Neural Induction
Matrigel
Expansion
Differentiation
day 0
5
10
1 dpi
4 dpi
B
Nes
Pax6
NesPax6DAPI
Day 10
C
MOCK
ZIKV
ZIKVACC3DAPI
CC3
12 dpi
12 dpi
D
MOCK
ZIKV
CC3+/total cells (%)
****
E
ZIKVANEG
ZIKVAPOS
Infection status of CC3+ cells (%)
F
MOCK
ZIKV
Lumen Area (µm2) in log2 scale
****
G
4 dpi
12 dpi
MOCK
HCMV-mNG 10⁵ PFU
HCMV-mNG 5x10⁵ PFU
BF
mNG
H
MOCK
HCMV-mNG
Organoid area (mm2)
ns
ns
Dose (PFU)
10⁵
5x10⁵
I
HCMV-mNG
mNG
mNG Sox1
4 dpi
J
MOCK
HCMV-mNG
DAPI mNG Sox1
12 dpi
12 dpi
K
ns
VZ Area (µm2) in log2 scale
MOCK
HCMV
L
4 dpi
8 dpi
MOCK
HSV-1
MOCK
HSV-1
10⁴ PFU
10³ PFU
M
MOCK
HSV-1(10⁴ PFU)
MOCK
HSV-1(10³ PFU)
Organoid area (mm2)
ns
****
*
****
4 dpi
8 dpi
4 dpi
8 dpi
N
MOCK
HSV-1
HSV1CC3DAPI
CC3
8 dpi
8 dpi
O
****
CC3+/total cells (%)
MOCK
HSV-1
P
****
Lumen Area (µm2) in log2 scale
MOCK
HSV-1
Q
ICP4 CC3 DAPI
8 dpi
R
Infection status of CC3+ cells (%)
ICP4NEG
ICP4POS

Figure S2

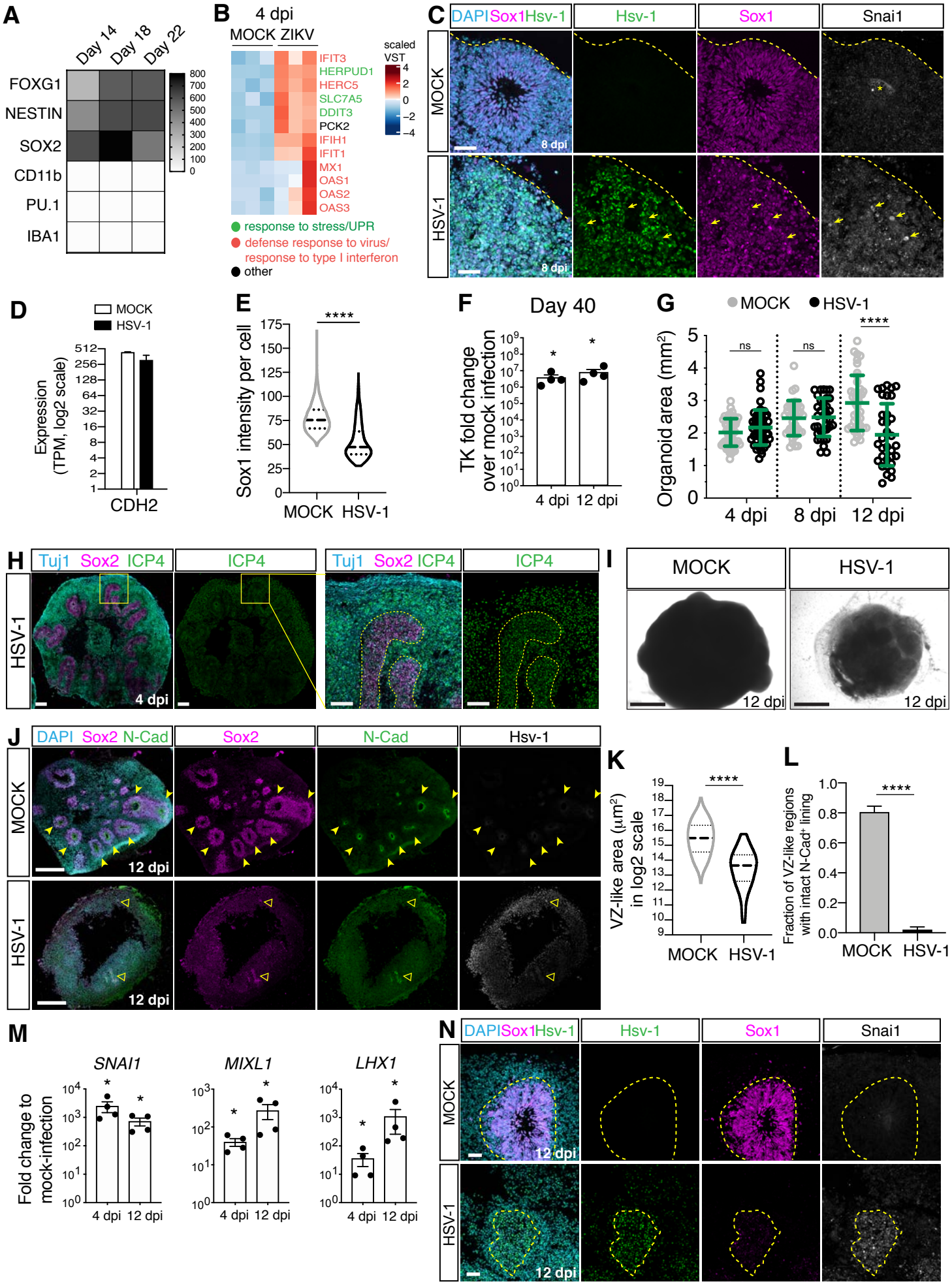

A
Day 14
Day 18
Day 22
FOXG1
NESTIN
SOX2
CD11b
PU.1
IBA1
800
700
600
500
400
300
200
100
0
B
4 dpi
MOCK ZIKV
scaled VST
4
2
0
−2
−4
IFIT3
HERPUD1
HERC5
SLC7A5
DDIT3
PCK2
IFIH1
IFIT1
MX1
OAS1
OAS2
OAS3
response to stress/UPR
defense response to virus/ response to type I interferon
other
C
DAPI Sox1 Hsv-1
Hsv-1
Sox1
Snai1
MOCK
HSV-1
8 dpi
8 dpi
D
MOCK
HSV-1
Expression (TPM, log2 scale)
512
256
128
64
32
16
8
4
2
1
CDH2
E
Sox1 intensity per cell
175
150
125
100
75
50
25
0
****
MOCK HSV-1
F
Day 40
TK fold change over mock infection
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
4 dpi 12 dpi
G
MOCK
HSV-1
Organoid area (mm^2)
5
4
3
2
1
0
ns
ns
****
4 dpi 8 dpi 12 dpi
H
Tuj1 Sox2 ICP4
ICP4
Tuj1 Sox2 ICP4
ICP4
HSV-1
4 dpi
I
MOCK
HSV-1
12 dpi
12 dpi
J
DAPI Sox2 N-Cad
Sox2
N-Cad
Hsv-1
MOCK
HSV-1
12 dpi
12 dpi
K
VZ-like area (μm^2) in log2 scale
19
18
17
16
15
14
13
12
11
10
9
****
MOCK HSV-1
L
Fraction of VZ-like regions with intact N-Cad+ lining
1.0
0.8
0.6
0.4
0.2
0.0
****
MOCK HSV-1
M
SNAI1
MIXL1
LHX1
Fold change to mock-infection
10^4
10^3
10^2
10^1
10^0
*
*
4 dpi 12 dpi
N
DAPI Sox1 Hsv-1
Hsv-1
Sox1
Snai1
MOCK
HSV-1
12 dpi
12 dpi

Figure S3

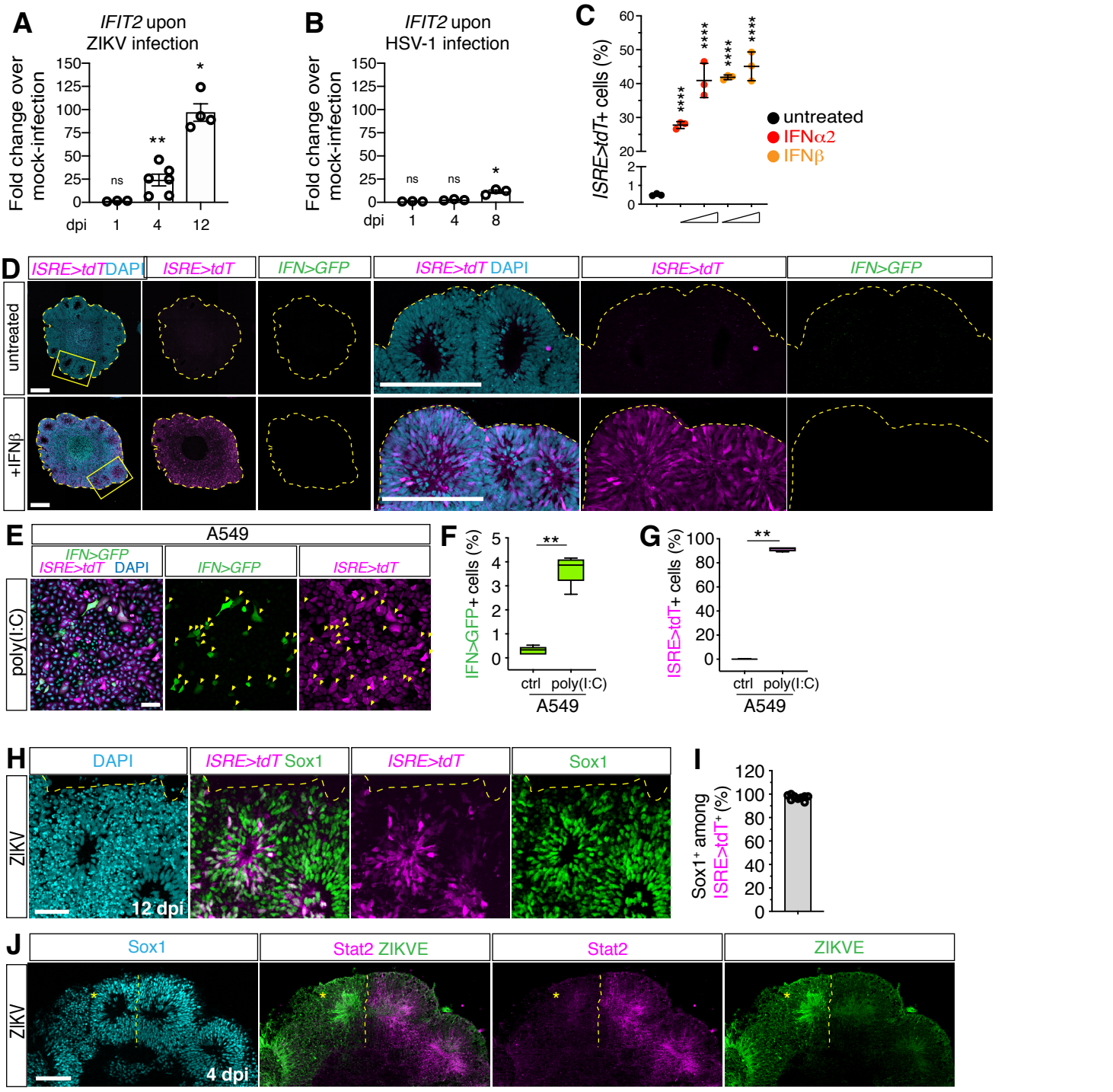

A
IFIT2 upon ZIKV infection
Fold change over mock-infection
dpi 1 4 12
B
IFIT2 upon HSV-1 infection
Fold change over mock-infection
dpi 1 4 8
C
ISRE>tdT+ cells (%)
untreated
IFNα2
IFNβ
D
ISRE>tdT DAPI
ISRE>tdT
IFN>GFP
untreated
+IFNβ
E
A549
IFN>GFP ISRE>tdT DAPI
poly(I:C)
F
IFN>GFP+ cells (%)
ctrl poly(I:C)
A549
G
ISRE>tdT+ cells (%)
ctrl poly(I:C)
A549
H
DAPI
ISRE>tdT Sox1
Sox1
ZIKV
12 dpi
I
Sox1+ among ISRE>tdT+ (%)
J
Sox1
Stat2 ZIKVE
Stat2
ZIKVE
ZIKV
4 dpi

Figure S4

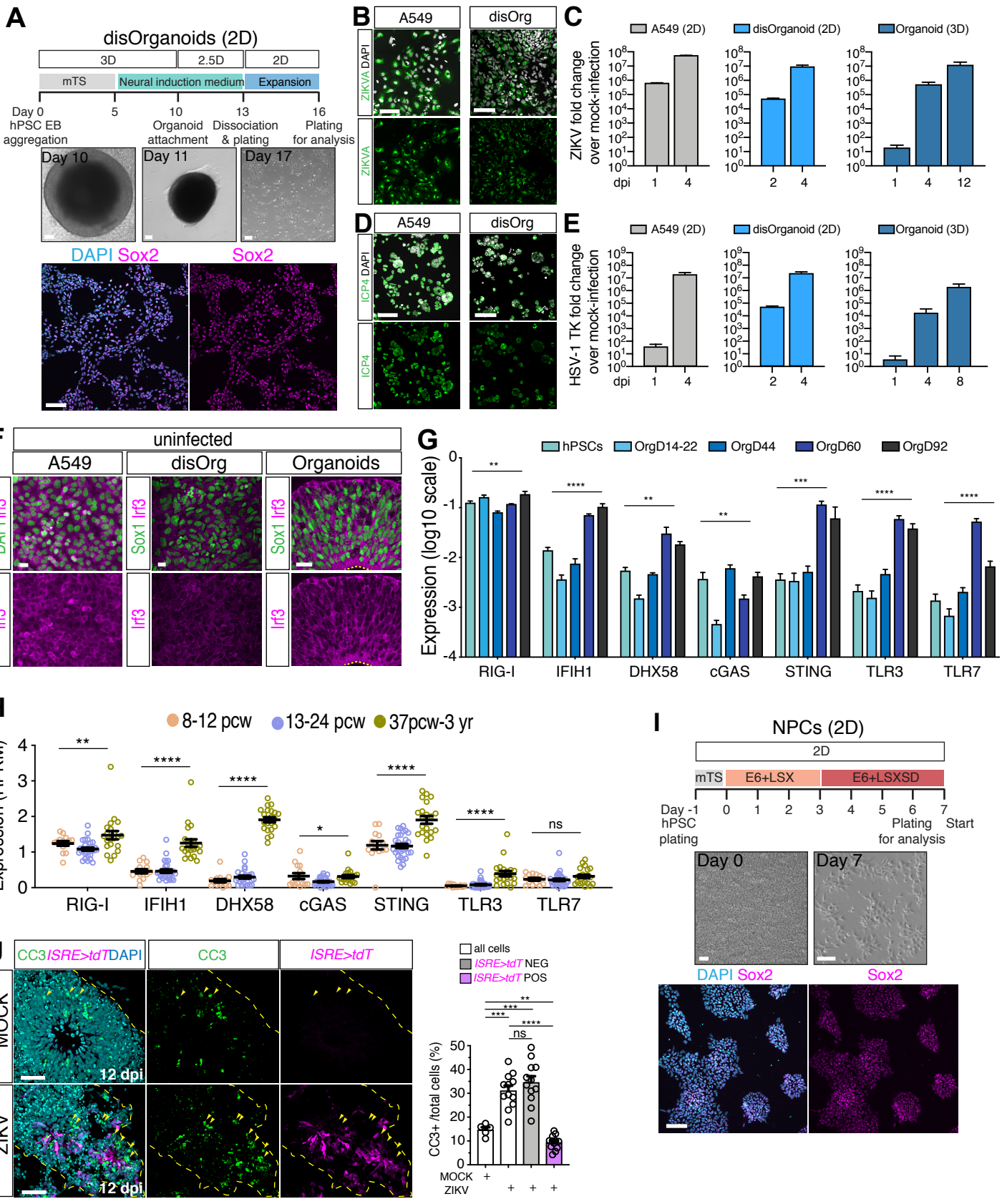
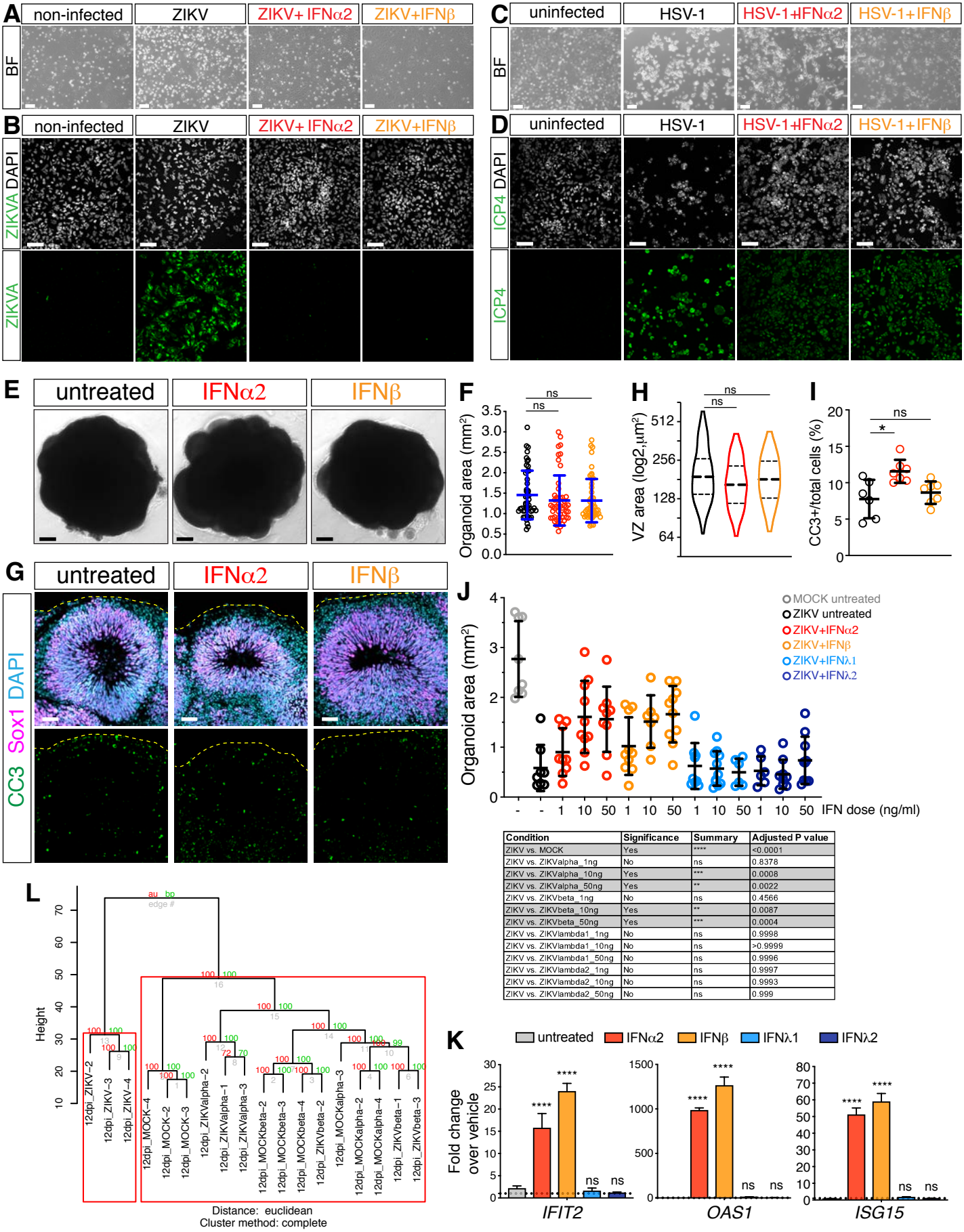

A
disOrganoids (2D)
3D
2.5D
2D
mTS
Neural induction medium
Expansion
Day 0 hPSC EB aggregation
5
10 Organoid attachment
13 Dissociation & plating
16 Plating for analysis
Day 10
Day 11
Day 17
DAPI Sox2
Sox2
B
A549
disOrg
ZIKVA DAPI
ZIKVA
C
A549 (2D)
disOrganoid (2D)
Organoid (3D)
ZIKV fold change over mock-infection
dpi
D
ICP4 DAPI
ICP4
E
HSV-1 TK fold change over mock-infection
F
uninfected
A549
disOrg
Organoids
DAPI Irf3
Sox1 Irf3
Irf3
G
hPSCs
OrgD14-22
OrgD44
OrgD60
OrgD92
Expression (log10 scale)
RIG-I
IFIH1
DHX58
cGAS
STING
TLR3
TLR7
H
8-12 pcw
13-24 pcw
37pcw-3 yr
Expression (RPKM)
ns
I
NPCs (2D)
2D
mTS
E6+LSX
E6+LSXSD
Day -1 hPSC plating
Plating
Start
for analysis
Day 0
Day 7
DAPI Sox2
Sox2
J
CC3 ISRE>tdT DAPI
CC3
ISRE>tdT
MOCK
ZIKV
12 dpi
all cells
ISRE>tdT NEG
ISRE>tdT POS
CC3+ /total cells (%)
MOCK
ZIKV

Figure S5



Condition	Significance	Summary	Adjusted P value
ZIKV vs. MOCK	Yes	****	<0.0001
ZIKV vs. ZIKValpha_1ng	No	ns	0.8378
ZIKV vs. ZIKValpha_10ng	Yes	***	0.0008
ZIKV vs. ZIKValpha_50ng	Yes	**	0.0022
ZIKV vs. ZIKVbeta_1ng	No	ns	0.4566
ZIKV vs. ZIKVbeta_10ng	Yes	**	0.0087
ZIKV vs. ZIKVbeta_50ng	Yes	***	0.0004
ZIKV vs. ZIKVlambda1_1ng	No	ns	0.9998
ZIKV vs. ZIKVlambda1_10ng	No	ns	>0.9999
ZIKV vs. ZIKVlambda1_50ng	No	ns	0.9996
ZIKV vs. ZIKVlambda2_1ng	No	ns	0.9997
ZIKV vs. ZIKVlambda2_10ng	No	ns	0.9993
ZIKV vs. ZIKVlambda2_50ng	No	ns	0.999

Figure S6

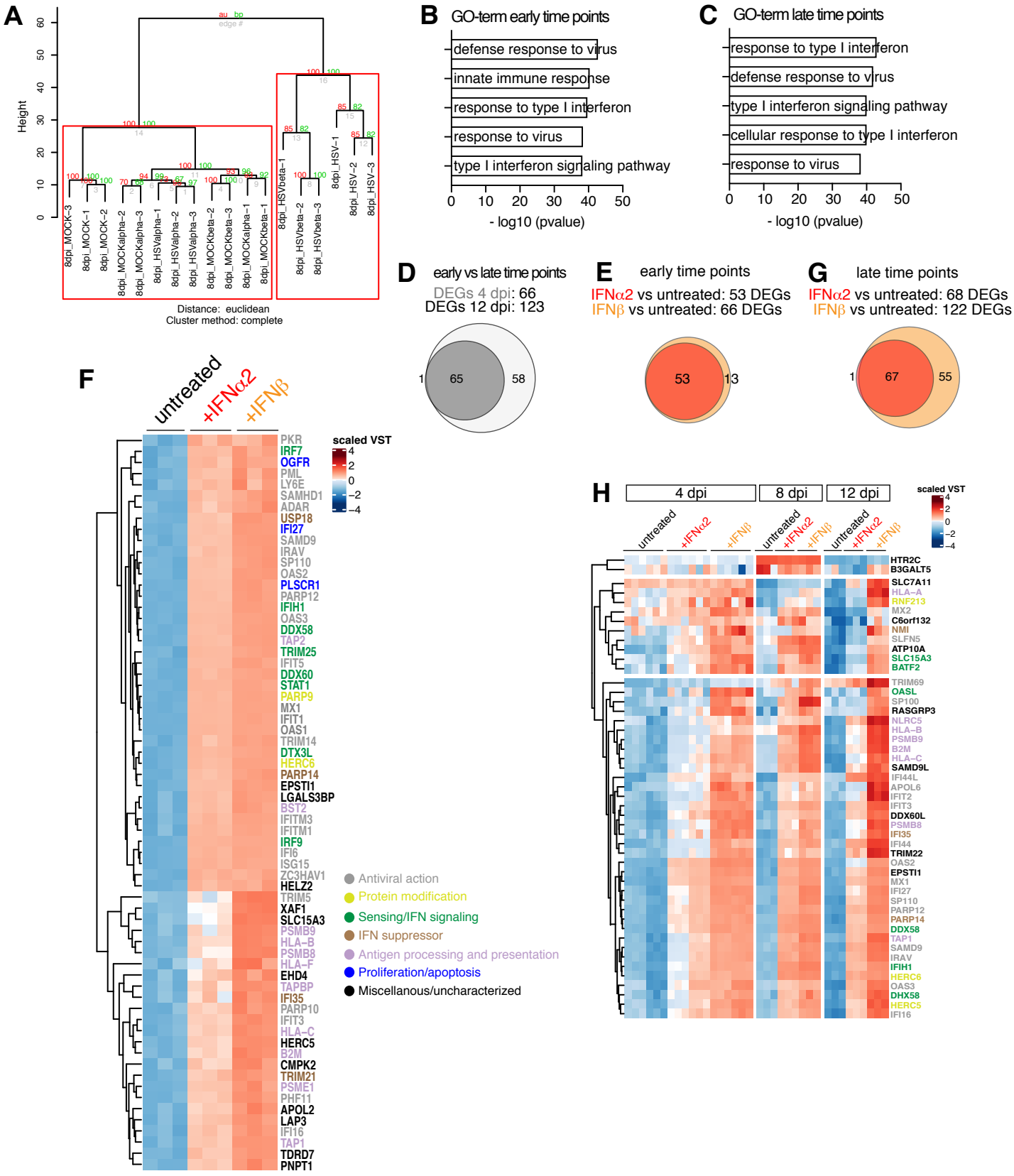
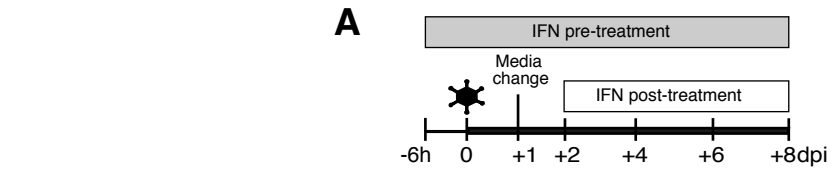
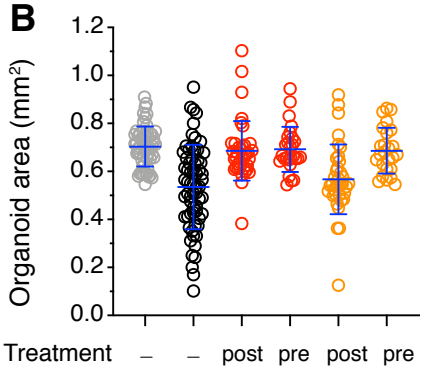
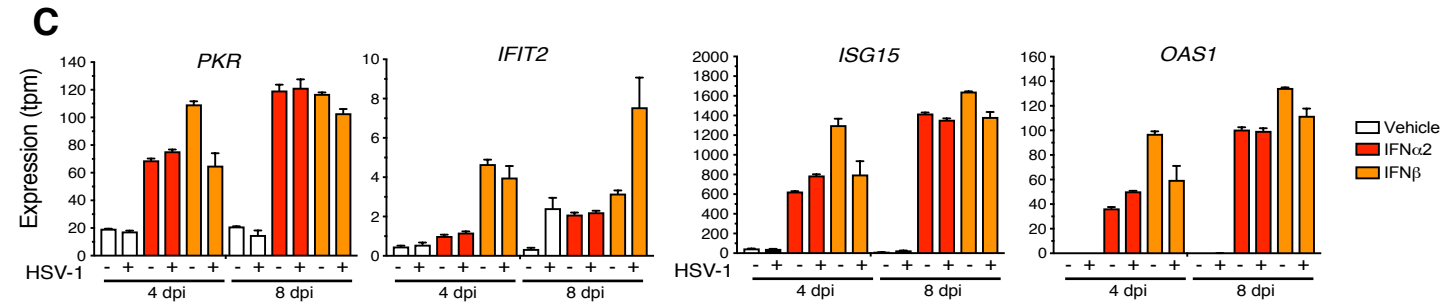

A
au bp
edge #
Height
8dpi_MOCK−3
8dpi_MOCK−1
8dpi_MOCK−2
8dpi_MOCKalpha−2
8dpi_MOCKalpha−3
8dpi_HSValpha−1
8dpi_HSValpha−2
8dpi_HSValpha−3
8dpi_MOCKbeta−2
8dpi_MOCKbeta−3
8dpi_MOCKalpha−1
8dpi_MOCKbeta−1
8dpi_HSVbeta−1
8dpi_HSVbeta−2
8dpi_HSVbeta−3
8dpi_HSV−1
8dpi_HSV−2
8dpi_HSV−3
Distance: euclidean
Cluster method: complete
B
GO-term early time points
defense response to virus
innate immune response
response to type I interferon
response to virus
type I interferon signaling pathway
- log10 (pvalue)
C
GO-term late time points
response to type I interferon
defense response to virus
type I interferon signaling pathway
cellular response to type I interferon
response to virus
- log10 (pvalue)
D
early vs late time points
DEGs 4 dpi: 66
DEGs 12 dpi: 123
E
early time points
IFNα2 vs untreated: 53 DEGs
IFNβ vs untreated: 66 DEGs
G
late time points
IFNα2 vs untreated: 68 DEGs
IFNβ vs untreated: 122 DEGs
F
untreated
+IFNα2
+IFNβ
scaled VST
Antiviral action
Protein modification
Sensing/IFN signaling
IFN suppressor
Antigen processing and presentation
Proliferation/apoptosis
Miscellanous/uncharacterized
H
4 dpi
8 dpi
12 dpi
scaled VST

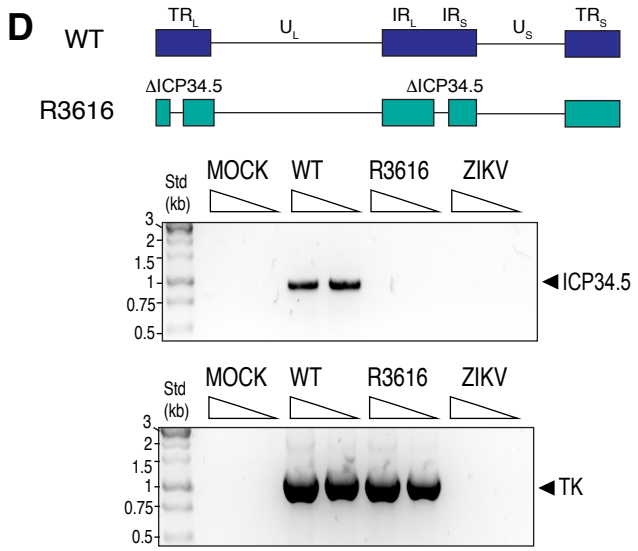
Figure S7

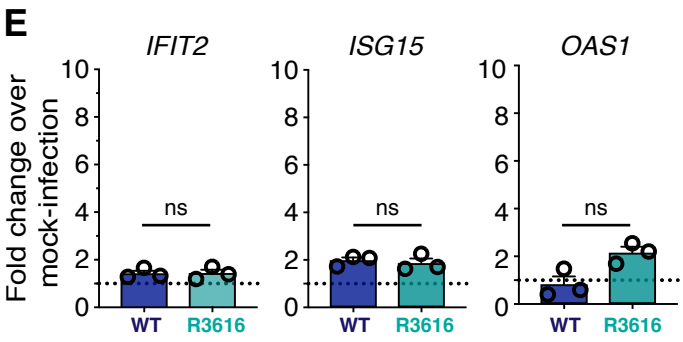




Comparison	Significance	adj P value
MOCK vs. HSV-1	***	<0.0001
MOCK vs. HSV-1+IFNα2 POST	ns	>0.9999
MOCK vs. HSV-1+IFNα2 PRE	***	<0.0001
MOCK vs. HSV-1 +IFNβ POST	***	<0.0001
MOCK vs. HSV-1+IFNβ PRE	ns	>0.9999
HSV-1 vs. HSV-1+IFNα2 POST	***	0.0002
HSV-1 vs. HSV-1+IFNβ POST	ns	>0.9999
HSV-1 vs. HSV-1+IFNα2 PRE	***	0.0002
HSV-1 vs. HSV-1+IFNβ PRE	***	0.0008
HSV-1+IFNα2 POST vs. HSV-1+IFNβ POST	**	0.0029
HSV-1+IFNα2 POST vs. HSV-1+IFNβ PRE	ns	>0.9999
HSV-1+IFNα2 POST vs. HSV-1+IFNα2 PRE	ns	>0.9999
HSV-1+IFNβ POST_vs. HSV-1+IFNβ PRE	**	0.0049
HSV-1+IFNβ POST vs. HSV-1+IFNα2 PRE	**	0.0014
HSV-1+IFNβ PRE vs. HSV-1+IFNα2 PRE	ns	>0.9999







SUPPLEMENTAL FIGURE LEGENDS

Figure S1. ZIKV and HSV-1 infections impair organoid growth (related to Figure 1)

A) Schematic diagram of viral exposure of 10-days-old brain organoids used in this study. See STAR Methods for details.

B) Immunostaining (scale bars 100 μm) of 10-days-old organoids showing neuroectodermal identity.

C-F) Immunostaining (scale bars 50 μm) of organoids exposed to ZIKV or MOCK-treated. Dashed lines indicate organoid surface based on DAPI signal. Shown in D is the quantification of apoptosis measured by the fraction of cleaved caspase 3 (CC3) positive cells over total cells at 12 dpi. Shown in E is the quantification of infection status marked by Zika virus antigen (ZIKVA) of apoptotic cells (CC3+) in ZIKV-exposed organoids. Values represent mean ± SEM (n=3 experiments with 21 regions from 12 MOCK organoids, n=17 regions from 14 ZIKV organoids; **** is $p<0.0001$, Mann-Whitney test). Quantification of the lumen area is shown in F. Violin plots show median and quartiles (n=129 regions in MOCK organoids, n=110 regions in infected organoids from 3 experiments; **** is $p<0.0001$, Mann-Whitney test).

G-H) Images (scale bars 200 μm) and area quantification of organoids exposed to Human Cytomegalovirus-mNeonGreen (HCMV-mNG) or MOCK-treated. Values are mean ± SD and represent individual organoids ($p>0.9999$ for 10^5 PFU; $p=0.8417$ for 5×10^5 PFU; Mann-Whitney test).

I-K) Immunostaining (scale bars 50 μm in I, 100 μm in J) and quantifications of the ventricular zone (VZ)-like area of organoids exposed to HCMV-mNG (5×10^5 PFU) or MOCK-treated and analyzed at 12 dpi. Dashed lines indicate organoid contour based on DAPI signal. Violin plots show median and quartiles (n=40 regions from 3 MOCK organoids, n=79 from 3 HCMV-mNG organoids; $p=0.2375$; Mann-Whitney test).

L-M) Images (scale bars 200 μm) and area measurements of organoids exposed to HSV-1 (10^4 and 10^3 PFU). Values are mean ± SD and represent individual organoids ($p=0.1397$ 10^4 PFU 4 dpi; $p=0.0164$ 10^3 PFU 4 dpi; **** is $p<0.0001$, Mann-Whitney test).

N-R) Immunostaining (scale bar 50 μm in N, 20 μm in Q) of organoids exposed to HSV-1 or MOCK-treated. Dashed lines indicate organoid surface based on DAPI signal. Arrowheads in Q indicate CC3+ cells. Note the altered nuclear morphology and peripheral chromatin of ICP4 positive cells. Infected cell polypeptide 4 (ICP4) is an immediate-early viral protein produced during HSV-1 lytic infection. Values in O represent mean ± SEM (n=3 experiments with 18 regions from 12 MOCK organoids and 16 regions from 12 HSV-1 organoids; **** is $p<0.0001$, Mann-Whitney test). Violin plots in P represent median and quartiles (n= 35 regions from 4 MOCK organoids and n=35 from 9 HSV-1 organoids from 3 experiments; **** is $p<0.0001$; Mann-Whitney test). Shown in R is the quantification of infection status (marked by ICP4) in

organoids exposed to HSV-1. Values represent mean ± SEM (n=3 experiments with a total of 16 regions from 12 organoids).

Dpi, days post-infection; ns, non-significant. See also Table S2. Pos, positive; Neg, negative.

Figure S2. ZIKV and HSV-1 infections elicit distinct transcriptional responses (related to Figure 2)

A) Heatmap showing high expression (>100 transcripts per million, tpm) of the neural progenitor markers *FOXG1*, *NESTIN* and *SOX2* in MOCK-treated organoids measured by RNA-sequencing. Low expression of the microglia/macrophage progenitor markers *CD11b*, *PU.1* and *IBA1* (<10 tpm) is shown for comparison.

B) Expression (in scaled Variance Stabilizing Transformation or VST) of differentially expressed genes in ZIKV-exposed vs MOCK-exposed organoids.

C) Immunostaining (scale bars 50 μm) of organoids exposed to HSV-1 or MOCK-treated. Dashed lines indicate organoid surface based on DAPI signal. Arrows indicate nuclear Snai1 signal in infected organoids, while the asterisk marks apical Sna1 signal in mock-treated organoids.

D) Expression (in transcripts per million, tpm) of the N-cadherin gene *CDH2* measured by RNA-sequencing at 8 dpi. Values are mean ± SD (n=3).

E) Quantification of Sox1 mean intensity per cell at 8 dpi. Values are median and quartiles (n=7961 cells from 4 MOCK organoids, n=5316 cells from 5 HSV-1 organoids; **** is p<0.0001, Mann-Whitney test).

F) RT-qPCR analysis of HSV-1 thymidine kinase (TK) gene expression in 40-days-old organoids exposed to HSV-1 over MOCK-treated samples. Values are mean ± SEM (n=4 experiments, p=0.0286, Mann-Whitney test).

G) Area quantification of 40-days-old organoids infected with HSV-1 or MOCK-treated. Values are mean ± SD and represent individual organoids (p=0.1846 4 dpi; p=0.7137 8dpi; **** is p<0.0001 12 dpi; Mann-Whitney test).

H) Immunostaining of 40-days-old organoids exposed to HSV-1. Dashed lines indicate ventricular zone (VZ) contour. Scale bars are 200 μm and 100 μm (insets).

I-L) Images, immunostaining (scale bars 500 μm) and quantifications of 40-days-old organoids analyzed at 12 dpi. Arrowheads and triangles in J indicate normal and disrupted ventricular zone (VZ)-like regions in MOCK-treated and HSV-1-infected organoids respectively. Violin plots in K show median and quartiles (n=128 regions from 12 MOCK organoids and n=86 regions from 12 HSV-1 organoids from 3 experiments; **** is p<0.0001, Mann-Whitney test). Data in L are mean ± SEM (n=3 experiments for a total of 18 regions for MOCK and 23 regions for HSV-1 organoids; **** is p<0.000,; Mann-Whitney test).

M) RT-qPCR analysis showing upregulation of the epithelial-mesenchymal transition (EMT) gene *SNAI1* and early mesodermal genes *MIXL1* and *LHX1* in 40-days-old organoids exposed to HSV-1 over MOCK-treated samples. Values are mean ± SEM (n=4 experiments, p=0.0286 in all cases, Mann-Whitney test over age-matched MOCK controls).
N) Immunostaining (scale bars 50 μm) of 40-days-old organoids exposed to HSV-1 or MOCK-treated. Dashed lines indicate VZ-like regions.
Dpi, days post-infection.

Figure S3. ZIKV and HSV-1 infections differentially engage the IFN-I system (related to Figure 3)
A-B) RT-qPCR analysis of *IFIT2* expression in organoids after ZIKV or HSV-1 exposure relative to their MOCK counterparts. Values are mean ± SEM (ZIKV: p=0.7 1 dpi; p=0.0022 4 dpi; p=0.0286 12 dpi; HSV-1: p=0.4 1 dpi; p=0.7 4 dpi; p=0.0119 8 dpi; Mann-Whitney test comparisons over age-matched MOCK-treated counterparts).
C) Quantification of *ISRE>tdTomato (ISRE>tdT)* positive cells measured by flow cytometry in 12-days-old organoids carrying the dual reporter system and incubated with increasing doses of the indicated recombinant IFN-I (10 ng/ml and 50 ng/ml) for one day. Measurements are mean ± SD (n=3; **** is p<0.0001, one-way ANOVA multiple comparisons over untreated).
D) Immunostaining (scale bars 200 μm) of 14-days-old organoids carrying the dual reporter system and incubated for 48 hours with IFNβ. Dashed lines indicate organoid contour based on DAPI signal. Insets show a magnified view. Note the low expression of *IFN>GFP* and *ISRE>tdT* reporters in the untreated sample.
E-G) Immunostaining (scale bars 100 μm) and quantifications of A549 cells engineered with the dual reporter system and analyzed 24 hours after stimulation with poly(I:C). Arrowheads indicate *IFN>GFP* positive cells. Graphs are Tukey plots (n=5; p=0.0043 in F, p=0.0043 in G, Mann-Whitney tests). Ctrl, control transfection.
H-I) Immunostaining (scale bars 50 μm) and quantification of *ISRE>tdT* expressing cells in ZIKV-exposed organoids engineered with the dual reporter system. Dashed lines indicate organoid surface based on DAPI signal. Values are mean ± SD (n=9 regions from 3 organoids).
J) Immunostaining (scale bars 100 μm) of organoids exposed to ZIKV. Dashed lines separate the infected region (marked by an asterisk) from the uninfected area. ZIKVE, Zika virus Envelope protein.
dpi, days post-infection; ns, non-significant.

Figure S4. The IFN-I response in brain organoids is more attenuated than in 2D cultures (related to Figure 4)

A) Outline of the protocol used to generate 2D cultures of cells dissociated from organoids (disOrganoids) derived from human pluripotent stem cells (hPSCs). Immunostaining (scale bars 100 μm) at day 16 is shown at the bottom. mTS, mTeSR1; EB, embryoid body. See STAR Methods for details.

B-E) Immunostaining (scale bars 100 μm) of A549 cells and disOrganoid cultures (disOrg) exposed to ZIKV or HSV-1 and analyzed at 4 dpi. Shown in C and E are the quantifications of ZIKV vRNA and HSV-1 thymidine kinase (TK) expression levels by RT-qPCR showing a much faster kinetics of ZIKV and HSV-1 replication in 2D cultures compared to brain organoids. Values are mean ± SEM (n=3 for A549, n=4 disOrg, n≥3 Org; p values for ZIKV: p=0.1 A549 1 and 4 dpi; p=0.0286 disOrg 2 and 4 dpi; p=0.1 Org 1 dpi, p=0.0022 Org 4 dpi, p=0.0286 Org 12 dpi; p values for HSV-1: p=0.1 A549; p=0.0286 disOrg; p>0.9999 Org 1 dpi, p=0.4 Org 4 dpi, p=0.0079 Org 12 dpi; Mann-Whitney tests). Note that HSV-1 infection in 2D cultures induce the formation of multicellular structures.

F) Immunostaining (scale bars 20 μm) of uninfected cultures showing cytoplasmic Irf3 localization.

G) Expression of nucleic acid sensors measured by RT-qPCR in hPSCs and organoids at various stages. D, day; Org, organoids. Values are mean ± SEM (n=4 for hPSCs, OrgD44, OrgD60; n=5 OrgD14-22; n=3 OrgD92; p=0.0024 RIG-I, p=0.0040 DHX58, p=0.0017 cGAS, p=0.0006 STING, **** is p<0.0001, one-way ANOVA).

H) Expression of nucleic acid sensors (in Reads Per Kilobase of transcript, per Million mapped reads, RPKM) in human fetal brains. Data were retrieved from the BrainSpan dataset (Miller et al., 2014) and include measurements from dorsolateral, ventrolateral and medial prefrontal cortex isolated from brains at various developmental ages. Values are mean ± SEM (p=0.0016 RIG-I; p=0.0101 cGAS; p=0.1921 TLR7; **** is p<0.0001, one-way ANOVA). pcw, post conceptional week; yrs, years.

I) Outline of the protocol used to generate human neural progenitor cells (NPCs) via monolayer cultures. Immunostaining (scale bars 100 μm) at day 7 is shown at the bottom. See STAR Methods for details.

J) Immunostaining (scale bars 50 μm) and quantification of apoptosis in organoids carrying the dual reporter system, exposed to ZIKV or MOCK-treated. Dashed lines indicate organoid surface based on DAPI signal. Arrowheads indicate examples of tdTomato-negative CC3+ cells. CC3+ cells were scored regardless of their *ISRE>tdT* expression (all cells) or based on *ISRE>tdT* positive (POS) or negative (NEG) expression. Values are mean ± SEM and represent individual regions from 3 organoids for MOCK and 4 for ZIKV (p=0.0002 ZIKV all vs

MOCK all; p=0.0002 *ISRE>tdT* NEG vs MOCK all; p=0.003 *ISRE>tdT* POS vs MOCK all; p=0.003 *ISRE>tdT* POS vs MOCK all; p=0.3 *ISRE>tdT* NEG vs ZIKV all; p<0.0001 *ISRE>tdT* POS vs ZIKV all; unpaired t tests).
Dpi, days post-infection.

Figure S5. Effect of IFN-I in 2D and 3D cultures (related to Figure 5)

A-D) Images and immunostaining (scale bars 100 μm) of 2D cultures of cells dissociated from organoids (disOrganoids) infected with ZIKV or HSV-1, treated with IFN-I and analyzed at 4 dpi. IFN-I were administered at 2 and 48 hours after exposure.

E-I) Images (in E, scale bars 200 μm) and immunostaining (in G, scale bars 50 μm) of uninfected organoids treated with IFN-I as described in Figure 5A and analyzed at 12 dpi. Dashed lines mark the organoid surface according to DAPI signal. Data in F are mean ± SD and represent individual organoids (p=0.3296 IFNα2 vs untreated; p=0.5560 IFNβ vs untreated, Kruskal-Wallis multiple comparisons tests). Violin plots in H show median and quartiles (n=105 regions from 7 untreated organoids, n=103 from 7 IFNα2-treated organoids, n=119 from 9 IFNβ-treated organoids; p=0.0907 for IFNα2 vs untreated; p>0.9999 for IFNβ vs untreated, Kruskal-Wallis multiple comparisons test). Values in I represent mean ± SD (n=6 untreated regions, n=7 IFNα2-treated regions, n=7 IFNβ-treated regions, from 2 organoids per condition; p=0.0218 IFNα2 vs untreated, p>0.9999 IFNβ vs untreated, Kruskal-Wallis multiple comparisons tests). See also Table S2.

J) Area quantification of organoids exposed to ZIKV and treated with increasing doses of IFN-I and type III IFNs (IFNλ1 and IFNλ2) as described in Figure 5A. Organoids were analyzed at 12 dpi. Values are mean ± SD and represent individual organoids (one-way ANOVA with Dunnett's multiple comparisons tests).

K) Quantification of ISG gene expression by RT-qPCR in 12-days-old organoids after incubation with the indicated interferons (IFNs) for one day. Values are mean ± SEM (n=3; *IFIT2*: p=0.9977 IFNλ1, p=0.9832 IFNλ2; *OAS1*: p=0.9992 IFNλ1; p>0.9999 IFNλ2; *ISG15*: p=0.9992 IFNλ1; p>0.9999 IFNλ2; **** is p<0.0001, one-way ANOVA with Tukey's multiple comparisons test to the untreated condition).

L) Dendrogram showing hierarchical clustering of ZIKV-infected organoids analyzed by RNA-sequencing. AU and BP values (%) are shown on the edges of the clustering. Red boxes indicate the main clusters identified with AU larger than 95%. AU, approximately unbiased; BP, bootstrap probability.

Dpi, days post-infection; ns, non-significant.

Figure S6. IFNβ treatment fails to prevent HSV-1-induced organoid defects (related to Figure 6)

A) Dendrogram showing hierarchical clustering of HSV-1-infected organoids treated with interferons as described in Figure 6A and analyzed by RNA-sequencing. AU and BP values (%) are shown on the edges of the clustering. Red boxes indicate the main clusters identified with AU larger than 95%. AU, approximately unbiased; BP, bootstrap probability.

B-D) Analysis of differentially expressed genes (DEGs) combined from IFNα2-treated and IFNβ-treated samples vs untreated controls at early and late time points (corresponding to 4 and 12 dpi respectively). The top 5 GO-terms are shown.

E-G) Analysis of differentially expressed genes (DEGs) in IFNα2-treated and IFNβ-treated samples vs untreated controls at early and late time points. VST, variance stabilizing transformation.

H) Time-resolved expression of genes differentially expressed in IFNα2- or IFNβ-treated samples.

dpi, days post-infection.

Figure S7. HSV-1 selectively counteracts IFNβ activity (related to Figure 7)

A-B) Outline of IFN-I pre- and post-treatment experiments and area quantification of organoids at 8 dpi. Lines are mean ± SD and represent individual organoids (Kruskal-Wallis multiple comparisons tests). See also Table S2.

C) Expression of the ISGs *PKR*, *IFIT2*, *ISG15* and *OAS1* measured by RNA-sequencing. Values represent mean ± SEM (n=3).

D) Schematic diagram of a linearized DNA molecule of HSV-1 showing the relevant features of wild type (WT) HSV-1 and the deletion of both copies of the ICP34.5 gene in the R3616 mutant virus. HSV-1 genome consists of two covalently joined segments, L (long) and S (short), each comprising a unique region (U) flanked by a set of terminal and inverted repeats (TR and IR). Bottom panels show the PCR amplification products for ICP34.5 and thymidine kinase (TK) sequences from viral nucleic acids preparations and analyzed by electrophoresis. Std, size standards in kilobases (kb).

E) Expression of ISGs measured by RT-qPCR analysis in organoids infected with HSV-1 wild type or R3616 (10^2 PFU) at 8 dpi. Data are mean ± SEM (n=3; p>0.9999 *IFIT2*; p=0.7 *ISG15*; p=0.1 *OAS1*; Mann-Whitney test).

dpi, days post-infection; ns, non-significant.

SUPPLEMENTAL ITEMS

Table S1. Summary of TORCH infection experiments in early-stage organoids (related to Figure 1, S1 and 7)

TORCH agent (strain)	Family (subfamily)	Genome	Genome size (kb)	Target cell	Dose	Replication	Organoid growth phenotype
ZIKV (French Polynesian)	Flaviviridae	ssRNA(+)	10.7	hNPC	10^5 $TCID_{50}$	efficient	attenuated
HCMV (TB40/E)	Herpesviridae (Betaherpesvirinae)	dsDNA	235	hNPC	10^5 PFU	inefficient	none
					5×10^5 PFU	inefficient	none
HSV-1 (F)	Herpesviridae (Alphaherpesvirinae)	dsDNA	152	hNPC	10^4 PFU	efficient	severely attenuated
					10^3 PFU	efficient	severely attenuated
					10^2 PFU	efficient	attenuated
HSV-1 R3616 (F)	Herpesviridae (Alphaherpesvirinae)	dsDNA	152	hNPC	10^3 PFU	efficient	attenuated
					10^2 PFU	inefficient	very mildly attenuated

Table S2. Summary of organoids used in various experiments (related to Figure 1, S1, S2, 5, S5, 6, 7 and S7)

Figure	Experiment	No. viral particles per organoid	No. organoid batches (experiments)	Total no. organoids
1B	ZIKV infection	10^5 $TCID_{50}$ units	5	n=35 MOCK 4 dpi n=32 ZIKV 4 dpi n=42 MOCK 8 dpi n=28 ZIKV 8 dpi n=46 MOCK 12 dpi n=51 ZIKV 12 dpi
S1H	HCMV	10^5 PFU	3	n=16 MOCK 12 dpi n=18 HCMV 12 dpi
S1H	HCMV	5×10^5 PFU	1	n=6 MOCK 12 dpi n=5 HCMV 12 dpi
S1M	HSV-1 infection	10^4 PFU	2	n=9 MOCK 4 dpi n=10 HSV-1 4 dpi n=13 MOCK 8 dpi n=14 HSV 8 dpi
S1M	HSV-1 infection	10^3 PFU	2	n=29 MOCK 4 dpi n=37 HSV-1 4 dpi n=25 MOCK 8 dpi n=32 HSV-1 8 dpi
1I	HSV-1 infection	10^2 PFU	3	n=58 MOCK 4 dpi n=74 HSV-1 4 dpi n=72 MOCK 8 dpi n=87 HSV-1 8 dpi
S2G	HSV-1 infection (day 40)	6×10^2 PFU	5	n=52 MOCK 4 dpi n=51 HSV-1 4 dpi n=40 MOCK 8 dpi n=35 HSV-1 8 dpi n=40 MOCK 12 dpi n=33 HSV-1 12 dpi
5C	IFN-I treatment against ZIKV	10^5 $TCID_{50}$ units	3	n=26 MOCK 12 dpi n=22 ZIKV 12 dpi n=29 ZIKV+IFNα2 12 dpi n=25 ZIKV+IFNβ 12 dpi
S5F	IFN-I treatment	none	5	n=49 untreated n=50 IFNα2 n=50 IFNβ
6C	IFN-I treatment against HSV-1	10^2 PFU	3	n=72 MOCK 8 dpi n=87 HSV-1 8 dpi n=54 HSV-1+IFNα2 8 dpi n=68 HSV-1+IFNβ 8 dpi
7K	IFN-I treatment against HSV-1 R3616	10^2 PFU	1	n=17 MOCK 8 dpi n=22 R3616 8 dpi n=11 R3616+IFNα2 8 dpi n=11 R3616+IFNβ 8 dpi
S7B	IFN-I pre and post treatment against HSV-1 WT	10^2 PFU	5	n=97 MOCK 8 dpi n=107 HSV-1 8 dpi n=40 HSV-1+postIFNα2 8 dpi n=44 HSV-1+preIFNα2 8 dpi n=37 HSV-1+postIFNβ 8 dpi n=41 HSV-1+preIFNβ 8 dpi

Table S3. Differential gene expression in infected versus MOCK organoids (related to Figure 2 and S2)

A-B) Differentially expressed genes (DEGs) in ZIKV-exposed versus MOCK-exposed organoids at 12 days post-infection (dpi, A) and in HSV-1-exposed versus MOCK-exposed organoids at 8 dpi (B).

Table S4. Primer sequences used for RT-qPCR (related to STAR Methods)

Target and sequences	SOURCE	IDENTIFIER
Primers TBP: forward 5->3 GGGCACCACTCCACTGTATC reverse 5->3 CGAAGTGCAATGGTCTTTAGG	This study	N/A
Primers IFNA: Forward 5->3 CGATGGCCTCGCCCTTTGCTTTA Reverse 5->3 GGGTCTCAGGGAGATCACAGCCC	(Paijo et al., 2016)	N/A
Primers IFNB1: Forward 5->3 TGTGGCAATTGAATGGGAGGCTTGA Reverse 5->3 TCAATGCGGCGTCCTCCTTCTG	(Paijo et al., 2016)	N/A
Primers ISG15: Forward 5->3 TGTCGGTGTCAGAGCTGAAG Reverse 5->3 AGAGGTTCGTCGCATTTGTC	This study	N/A
Primers IFIT2: Forward 5->3 CAGCTGAGAATTGCACTGCAA Reverse 5->3 CGTAGGCTGCTCTCCAAGGA	This study	N/A
Primers OAS1: Forward 5->3 TGACTGGCGGCTATAAACC Reverse 5->3 TGGGCTGTGTTGAAATGTGT	This study	N/A
Primers RIG-I: Forward 5->3 AGTGAGCATGCACGAATGAA Reverse 5->3 GGGATCCCTGGAAACACTTT	(Hamel et al., 2015)	N/A
Primers IFIH1: Forward 5->3 GCCATTGCAGATGCAACCAG Reverse 5->3 TTGCGATTTCCTTCTTTTGCAG	(Hamel et al., 2015)	N/A
Primers DHX58: Forward 5->3 GCCCTCGGGGTATCATCTTC Reverse 5->3 CCCGGATGTCCACAGTCTG	Primer Bank	149408121c3
Primers TLR3: Forward 5->3 TTGCCTTGTATCTACTTTTGGGG Reverse 5->3 TCAACACTGTTATGTTTGTGGGT	Primer Bank	19718735c1
Primers TLR7: Forward 5->3 TCCTTGGGGCTAGATGGTTTC Reverse 5->3 TCCACGATCACATGGTTCTTTG	Primer Bank	67944638c1
Primers cGAS: Forward 5->3 CCCAAGCATGCAAAGGAAGG Reverse 5->3 ACAATCTTTCCTGCAACATTTCT	(Paijo et al., 2016)	N/A
Primers STING: Forward 5->3 CACCTGTGTCCTGGAGTACG Reverse 5->3 CATCTGCAGGTTCCTGGTAGG	(Paijo et al., 2016)	N/A
Primers TK: Forward 5->3 ACCCGCTTAACAGCGTCAACA Reverse 5->3 CCAAAGAGGTGCGGGAGTTT	(Ferenczy and DeLuca, 2009)	N/A
Primers ACTIN: Forward 5->3 AAATCTGGCACCACACCTTC Reverse 5->3 AGAGGCGTACAGGGATAGCA	(Lancaster et al., 2013)	N/A
Primers SNAI1: Forward 5->3 TCGGAAGCCTAACTACAGCGA Reverse 5->3 AGATGAGCATTGGCAGCGAG	Primer Bank	301336132c1
Primers MIXL1: Forward 5->3 GGCGTCAGAGTGGGAAATCC Reverse 5->3 GGCAGGCAGTTCACATCTACC	Primer Bank	13994334c1
Primers LHX1: Forward 5->3 CCTGGACCGCTTTCTCTTGAA Reverse 5->3 ACCGAAACACCGGAAGAAGTC	Primer Bank	314122156c1
Primers ZIKV: ZIKA835 5->3 TTGGTCATGATACTGCTGATTGC ZIKA911c 5->3 CCTTCCACAAAGTCCCTATTGC	(Lanciotti et al., 2008)	N/A